

Positive Selection Procedure for Entrapment of Insertion Sequence Elements in Gram-Negative Bacteria

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We constructed the broad-host-range plasmid pUCD800 containing the *sacB* gene of *Bacillus subtilis* for use in the positive selection and isolation of insertion sequence (IS) elements in gram-negative bacteria. Cells containing pUCD800 do not grow on medium containing 5% sucrose unless the *sacB* gene is inactivated. By using pUCD800, we isolated a 1.4-kilobase putative IS element from *Agrobacterium tumefaciens* NT1RE by selection for growth on sucrose medium. This putative IS element appears to be unique to *Agrobacterium* strains.

Insertion sequence (IS) elements are mobile genetic elements that contain no detectable genes unrelated to insertion function and are generally shorter than 2 kilobases (kb) (2). Positive genetic selection of these elements is therefore not possible. IS elements were indirectly detected by their ability to cause spontaneous mutations in *Escherichia coli* and by the presence of additional DNA segments when DNA molecules were physically analyzed (8, 11, 13, 17, 22). IS elements were also detected as repetitive DNA segments by electron microscopic studies (4, 12, 19). These elements can be identified by sequence homology to a known IS element and by their ability to translocate to another replicon, resulting in the silencing of a gene, replicon fusion, or inversion.

Generally, the detection and isolation of a suspected IS element have been tedious. The development of a broad-host-range vector system utilizing the origin of DNA replication of the IncW plasmid pSa (3, 25) and the isolation of the structural gene *sacB* from *Bacillus subtilis* (10) have made it possible to design a means to positively select IS elements in gram-negative bacteria. The *sacB* gene encodes levansucrase (sucrose:2,6- β -D-fructan 6- β -D-fructosyltransferase; EC 2.4.1.10), a 50-kilodalton enzyme secreted in culture medium by *B. subtilis* after induction by sucrose. The enzyme catalyzes transfructorylation from sucrose to various acceptors, which results in two main physiological reactions: (i) levan synthesis and (ii) sucrose hydrolysis. We have discovered that the production of levansucrase in *E. coli*, *Agrobacterium tumefaciens*, and *Rhizobium meliloti* is lethal in the presence of 5% sucrose in agar medium, causing lysis within 1 h or inhibition of growth. Spontaneous insertions or deletions in *sacB* can be detected by inactivation of the *sacB* gene (24). Hence, a plasmid vector with broad-host-range features and bearing the *sacB* gene has been useful in the isolation of IS elements in gram-negative bacteria.

The *B. subtilis* *sacB* gene with its regulatory sequence *sacR* was originally isolated in pBR325 (10) and was subcloned in pLS306, a derivative of pJH101 (7). The *sacB* gene was inserted between the *Bam*HI and *Pst*II sites in the broad-host-range cosmid pUCD5 (3), resulting in the recombinant plasmid pUCD800 (Fig. 1). pUCD800 is suitable for the cloning of large fragments, using either the *Km*^r gene, by

screening for sensitivity to kanamycin, or the *sacB* gene, by selecting for resistance to sucrose. Like pUCD5, pUCD800 can be transferred by transformation (16) or by conjugation, promoted by triparental matings with pRK2013 (5), to various gram-negative bacteria. In *A. tumefaciens* NT1RE, a pTiC58 plasmid-free derivative that is resistant to rifampin and erythromycin (15), strains B6, Ach-5, and 1D1; and *R. meliloti* 102F34 Nal^r (from R. Valentine) the production of levansucrase as determined by an enzymatic assay (10) was only detected in strains carrying pUCD800 and proved lethal when cells were plated on LB agar (18) containing 5% sucrose. With this feature, pUCD800 was useful in the isolation of IS elements.

A. tumefaciens NT1RE cells (5×10^8 CFU/ml) containing pUCD800 were plated on LB medium containing 5% sucrose (the sucrose was filter sterilized separately and then added to the medium). In one test, 22 colonies that appeared on the agar medium were individually scored by agarose gel electrophoresis for the presence of pUCD800 containing an insert. Those containing a putative IS element (5 of the 22) were selected, and 1 of these, designated pUCD800-18, was chosen for further study. pUCD800-18 DNA prepared by the method of Kao et al. (15) was transformed into *E. coli* HB101 and subsequently purified by using a modification of the procedure of Bolivar et al. (1). The latter procedure was used for the isolation of pUCD800 DNA from *E. coli*. The size of the insert was estimated by agarose gel electrophoresis, using *Hind*III- and *Eco*RI-digested lambda DNA standards. Restriction mapping showed that pUCD800-18 contained an insert of approximately 1.4 kb in the *sacB* gene (Fig. 2).

To determine whether the insertion in pUCD800-18 originated from the chromosome of *A. tumefaciens* NT1RE, pUCD800 and pUCD800-18 were labeled with [³²P]dATP and [³²P]dCTP by nick translation with DNA polymerase I (20) and used as hybridization probes. Chromosomal DNA from *E. coli* HB101, *A. tumefaciens* NT1RE, and other test bacteria (described below) were isolated as described previously (9). Ti plasmid DNA was purified as described previously (15). The DNA samples were denatured, bound on nitrocellulose membranes (type NC; Schleicher & Schuell Inc., Keene, N.H.), and subjected to spot hybridization as described by Kafatos et al. (14), with the exception that a slot manifold was used (Schleicher & Schuell). The chromosomal DNA of NT1RE hybridized strongly (as judged by the intensity of the autoradiograph) to pUCD800-18 but not to

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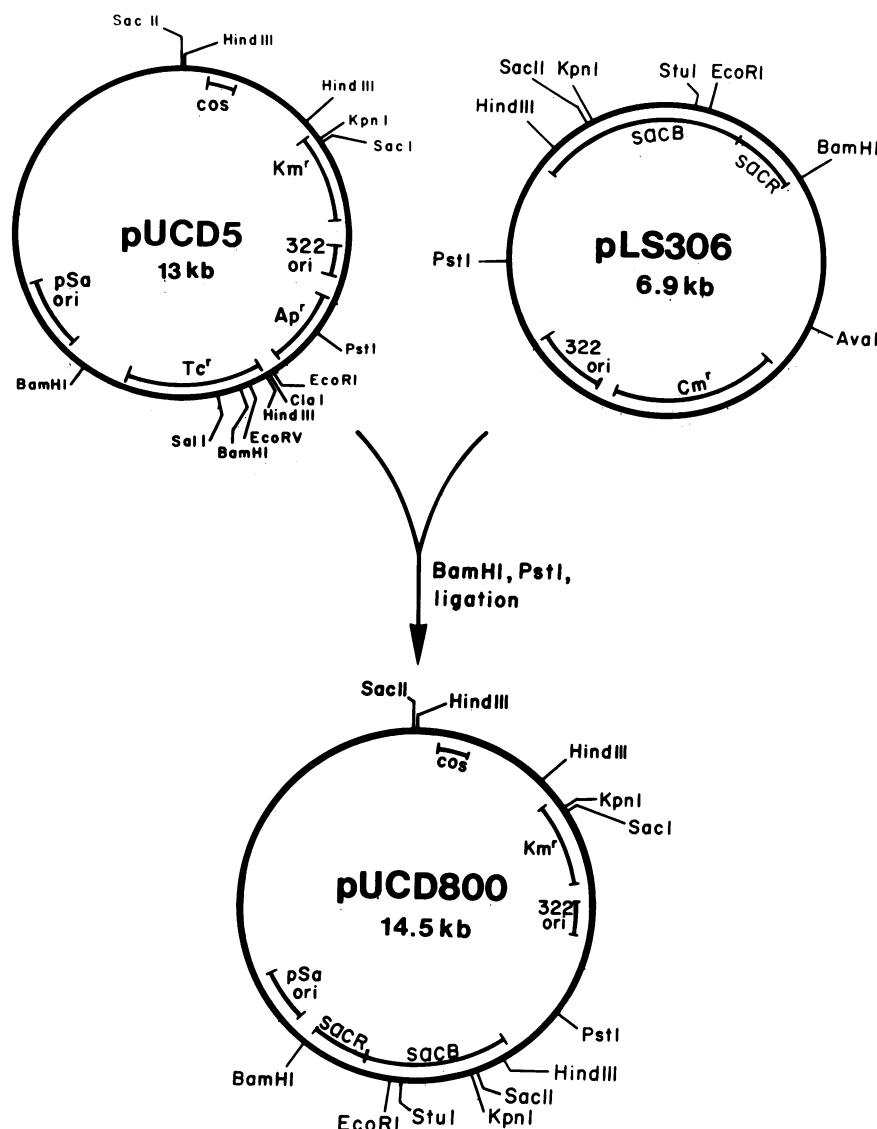


FIG. 1. Construction of IS-element-trapping vector pUCD800. The 2.6-kb *sacB* gene and the regulatory unit *sacR* were removed from pLS306 by digestion with *Pst*I and *Bam*HI. The broad-host-range cosmid pUCD5 DNA was digested with *Pst*I and *Bam*HI, which removed part of the tetracycline resistance (*Tc*^r) gene and the ampicillin resistance (*Ap*^r) gene. Ligation yielded a recombination molecule consisting of pUCD5 lacking the *Tc*^r gene and part of the *Ap*^r gene and containing *sacB* and *sacR*.

pUCD800 (Fig. 3). As expected, both probes hybridized strongly to the chromosomal DNA of *E. coli* HB101 since pUCD800 also carries a remnant of *IS1* (24) and intact *IS1* elements are found in the *E. coli* chromosome (21, 23). The failure of pUCD800 to hybridize to *A. tumefaciens* NT1RE DNA suggests that *IS1* sequences are not present in strain NT1RE. On the other hand, the insertion that is present in the *sacB* gene of pUCD800-18 hybridized strongly to the chromosomal DNA of NT1RE as well as to that of *A. tumefaciens* C58, B6(806), and 1D1119, and weakly to that of *A. tumefaciens* LBA4301 and 12D12 (Fig. 3B). No hybridization was detected above background levels between pUCD800-18 and the chromosomal DNAs of *Corynebacterium michiganense*, *Erwinia amylovora*, *Xanthomonas campestris* pv. *malvacearum*, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *tomato*, and *P. syringae* pv. *lachrymans* (compare lane 6b

with lane 7b in Fig. 3B). These data suggest that the putative IS element isolated from NT1RE is confined to *Agrobacterium* strains.

Based on the restriction analyses (Fig. 2 and data not shown), the putative IS element in pUCD800-18 is located to the left of the unique *Kpn*I site in *sacB* (Fig. 1). To test whether this element is indeed within *sacB*, 50 µg of pUCD800-18 was digested with 100 U of *Sac*II in 500 µl of low-salt digestion buffer (10 mM MgCl₂, 10 mM Tris chloride [pH 7.5], 1 mM dithiothreitol) at 37°C. After 3 h, the solution was brought to 100 mM NaCl, and 100 U of *Bam*HI was added. The mixture was then incubated at 37°C overnight. The double digestion produced three fragments, the smallest of which contained the *sacR-sacB* insert of 3 kb based on the restriction analysis (Fig. 2). These fragments were separated electrophoretically in a 0.7% agarose gel, and the 3-kb fragment was collected electrophoretically (6) on a DEAE

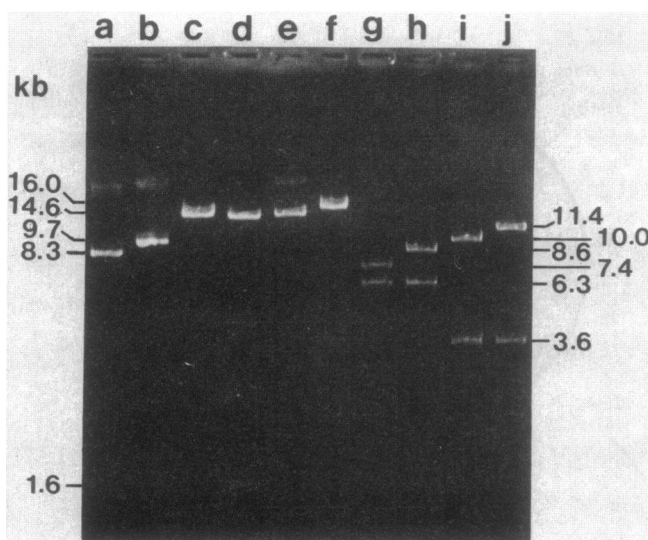


FIG. 2. Agarose gel electrophoresis of pUCD800 and pUCD800-18 DNA digested with the following restriction enzymes: undigested pUCD800 (lane a) and pUCD800-18 (lane b) (with open circular types above the major band in these lanes); pUCD800 and pUCD800-18 digested, respectively, with *Pst*I (lanes c and d), *Sac*I (lanes e and f), *Sac*II (lanes g and h), and *Kpn*I (lanes i and j).

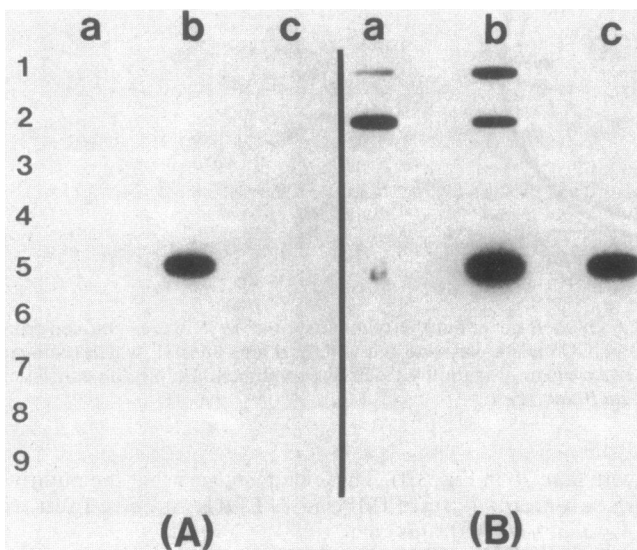


FIG. 3. Slot blot hybridizations with radiolabeled pUCD800 (section A) and pUCD800-18 (section B) as probes. Chromosomal DNAs (10 μ g per slot) of the following strains were analyzed in slots: a1, *A. tumefaciens* LBA4301; a2, *A. tumefaciens* B6(806); a3, blank; a4, *C. michiganense*; a5, *X. campestris* pv. *malvacearum*; a6, blank; a7, *P. syringae* pv. *morsprunorum*; a8, *P. syringae* pv. *lachrymans* 3; a9, *P. syringae* pv. *morsprunorum* 6-2; b1, *A. tumefaciens* C58; b2, *A. tumefaciens* 1D1119; b3, blank; b4, *P. syringae* pv. *syringae*; b5, *E. coli* HB101; b6, blank; b7, *P. syringae* pv. *lachrymans* 4; b8, *P. syringae* pv. *tomato* 3; b9, *P. syringae* pv. *lachrymans* 5; c1, *A. tumefaciens* 12D12; c2, pTiC58; c3, blank; c4, *E. amylovora*; c5, *A. tumefaciens* NT1RE; c6, blank; c7, *P. syringae* pv. *tomato*; c8, *P. syringae* pv. *morsprunorum* 4; c9, *P. syringae* pv. *tomato* 1-2.

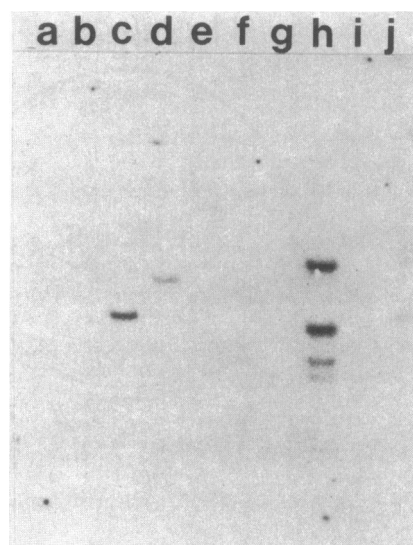


FIG. 4. Blot hybridization of chromosomal DNA digested with *Pst*I and resolved by agarose gel electrophoresis. Chromosomal DNAs of the following strains were analyzed by using a 3-kb fragment containing the 1.4-kb *A. tumefaciens* IS element as a probe: *R. meliloti* 102F34 (lane a), *A. tumefaciens* LBA4301 (lane b), *A. tumefaciens* C58 (lane c), *A. tumefaciens* 1D1119 (lane d), *A. tumefaciens* B6(806) (lane e), *A. tumefaciens* 56A2R (lane f), *E. coli* HB101 (lane g), *A. tumefaciens* NT1RE (lane h), *X. campestris* pv. *malvacearum* (lane i), pTiC58 DNA alone (lane j).

membrane filter (type NA-45; Schleicher & Schuell). The 3-kb fragment was radiolabeled by nick translation (20) and used as the hybridization probe for blot hybridization (27) of chromosomal DNA that was digested with *Pst*I. Hybridization bands (Fig. 4) were noted with the following strains of *Pst*I-digested chromosomal DNA of *A. tumefaciens*: octopine strain LBA4301 (lane b, with weak band), nopaline strain C58 (lane c), grapevine strain 1D1119 (lane d), octopine strain B6(806) (lane e, with a barely detectable band), and avirulent strain NT1RE (lane h). A weak band was noted for pTiC58 DNA (lane j). The chromosomal DNAs of other bacteria showed no hybridizations with the 3-kb probe, which contains the 1.4-kb *A. tumefaciens* insertion in the *sacB* gene. Interestingly, unlike the other *A. tumefaciens* strains, there are four bands detected in the chromosomal DNA of NT1RE, suggesting the presence of this sequence in at least four different locations. Confirmation that the insertion in pUCD800-18 contains the structural features typical of an IS element awaits genetic translocation studies and nucleotide sequence analysis.

The positive method of selecting IS elements as exemplified by the retrieval of a putative uncharacterized IS element from strain NT1RE by the use of pUCD800 should facilitate isolation of IS elements from various gram-negative bacteria.

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